METABOLISM AND NUTRITION

Characterization of the Chicken Small Intestine Type IIb Sodium Phosphate Cotransporter[1]

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ABSTRACT Intestinal absorption and renal resorption play a critical role in overall phosphorus homeostasis in chickens. Using RNase-ligase-mediated rapid amplification of cDNA ends PCR, we obtained a cDNA from the broiler small intestine that encodes a type IIb Na-dependent phosphate transporter. The cDNA has an open reading frame of 2,022 bp and predicts a 674-amino acid protein with a molecular mass of approximately 74 kDa. Prediction of membrane spanning domains based on the hydrophilic and hydrophobic properties of the amino acids suggests 8 transmembrane domains, with both the NH_2 and COOH termini being intracellular. The Na-inorganic phosphate (Pi) IIb cotransporter has relative high homology with other type II Na-Pi cotransporters but low homology with the type I or type III Na-Pi cotransporters. Northern blot analysis demonstrated the presence of a single mRNA transcript present predominantly in the small intestine, with the highest expression in the duodenum, followed by the jejunum and ileum. In situ hybridization indicated that the Na-Pi cotransporter mRNA is expressed throughout the vertical crypt-villus axis of the small intestine. Reduction of P in the diet of chicks from hatch to 4 d of age resulted in a significant induction of Na-Pi cotransporter mRNA expression in the small intestine. Further study is needed to elucidate its physiological role in intestinal phosphate absorption in chickens.

Key words: phosphate, transporter, cloning, small intestine, chicken

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INTRODUCTION

Phosphorus is an essential nutrient and possesses numerous functions, both structural and metabolic, in all animals. Whole-body P homeostasis depends on a balance of intestinal absorption and renal excretion. Renal resorption of inorganic phosphate (**Pi**) is under tight physiological control and can readily adjust to altered P conditions, whereas the small intestine adapts to altered P conditions relatively slowly (Murer et al., 2000). Since the 1990s, several cDNA encoding Na-dependent P transporters (Na-Pi cotransporters) have been identified and classified into type I, type II, and type III based on sequence homology (Takeda et al., 1999). The type I Na-Pi cotransporter was first identified from the rabbit kidney cortex (Werner et al., 1991) and then found in humans, mice, rats, etc. (Chong et al., 1993, 1995; Li and Xie, 1995; Miyamoto et al., 1995). The type I Na-Pi cotransporter is not specific for Na-dependent Pi transport, because evidence for anion channel function with permeability for chloride and different organic anions was reported (Busch et al., 1996; Broer et al., 1998). The precise physiological role of the type I Na-Pi cotransporter is not well defined, and the characteristics of the Na-dependent Pi transport induced by this type of Na-Pi cotransporter suggests that it is not a major player in meditating brush-border membrane Na-Pi cotransport.

The type II Na-Pi cotransporter proteins are involved in regulating both intestinal Pi absorption and renal Pi resorption. Based on structure, tissue distribution, and pH dependency, the type II Na-Pi cotransporter is further subdivided into type IIa and type IIb (Hilfiker et al., 1998; Werner and Kinne, 2001). The type IIa Na-Pi cotransporter is primarily expressed in the apical membranes of the epithelial cells in the renal proximal tubules (Magagnin et al., 1993; Biber et al., 1996) and represents the predominant Na-Pi cotransporter in the kidney. The type IIb cotransporter is primarily expressed in the brush-border membranes of the small intestinal epithelium, where it is considered to be the major Na-Pi cotransporter (Hilfiker et al., 1998). The type IIb cotransporter is also expressed in the lung and secretory tissues (Feild et al., 1999). The type IIa Na-Pi cotransporters are pH-dependent, with basic conditions significantly facilitating the cotransport, whereas the type IIb cotransporters are less pH-dependent, and the

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transport activity is slightly higher at more acidic pH values (Hilfiker et al., 1998).

The type III Na-Pi cotransporters were originally identified as receptors for gibbon ape leukemia virus (Glvr-1) and mouse amphotropic retrovirus (Ram-1), and only later were they shown to mediate Na-Pi cotransport activity after expression experiments in *Xenopus* oocytes (Kavanaugh et al., 1994; Olah et al., 1994; Kavanaugh and Kabat, 1996). The expression of the type III Na-Pi cotransporter appears to be ubiquitous and is considered a housekeeping Pi transport system.

Although renal resorption of Pi has been extensively studied (Murer et al., 2000; Karim-Jimenez et al., 2001; Lambert et al., 2001; Traebert et al., 2001; Kohler et al., 2002), intestinal absorption of Pi has drawn considerably less attention. To understand whole-body P homeostasis, elucidation of P entry into the body in the small intestine is necessary. The efficiency of Pi absorption in the small intestine is low in chickens. For broilers fed diets containing normal levels of P and Ca, apparent ileal absorption of P is only about 40 to 50% (Ravindran et al., 2000; Tamin et al., 2004). The mechanisms of Pi absorption in the small intestine are ill-defined, having both the transcellular and paracellular pathways shown to be involved in translocation of Pi from the intestinal lumen to the serosal side of the intestine (Peterlik et al., 1981). The transcellular Pi transport is Na-dependent and consists of 3 steps: 1) entry into the intestinal epithelial cells at the brush-border membrane, 2) movement across the cell, and 3) exit at the basolateral membrane. Entry at the brush-border membrane occurs against a chemical concentration difference and an electrical potential difference and is an energy requiring an "uphill" process that requires Na (Danisi and Straub, 1980; Kinne, 1981). The luminal entry is considered the rate-limiting step for transcellular Pi transport (Berner et al., 1976; Hoffman et al., 1976; Danisi et al., 1981) and has been the focus of the majority of research on Pi transport in the small intestine.

The molecular identity of the Na-dependent transport system in the intestine remained unknown until a type IIb Na phosphate cotransporter (Na-Pi IIb) was identified in the brush-border membrane of the mouse small intestine (Hilfiker et al., 1998). The type IIb Na-Pi cotransporter was soon identified in humans, rats, and goats (Feild et al., 1999; Xu et al., 1999; Hashimoto et al., 2000; Huber et al., 2000). In rats and mice, it was demonstrated that Na-dependent Pi transport by brush-border membrane vesicles decreased with age, and Na-Pi IIb gene expression also decreased proportionally with age (Arima et al., 2002; Xu et al., 2002). It was also shown in mice and goats that this transporter played an important role in the adaptation of Pi transport to a low-Pi diet, mediated through a post-transcriptional process (Hattenhauer et al., 1999; Huber et al., 2002). Furthermore, recent studies in vitamin D receptor-deficient mice have shown that the expression of the transporter is sensitive to low Pi-containing diets and is independent of vitamin D (Segawa et al., 2004). The molecular basis of P transport in the small intestine of chickens has not been characterized.

The identification and characterization of a chicken homolog for the Na-Pi IIb cotransporter will provide a better understanding of Pi absorption mechanisms in the chicken and a target gene for manipulation to increase the birds' ability to absorb P. This study was conducted to describe the type IIb Na-Pi cotransporter in the chicken small intestine and determine its response to dietary P manipulation.

MATERIALS AND METHODS

RNA Extraction and Reverse Transcription-PCR

Total RNA was extracted from the duodenum, jejunum, and ileum of 28-d-old male Ross 308 broilers using Trizol reagent (Gibco BRL, Life Technologies, Rockville, MD) according to the manufacturer's instructions and resuspended in diethylpyrocarbonate-treated water. Concentration and quality of the RNA were determined by measuring the absorbance at 260 nm and agarose gel electrophoresis, respectively. To verify the presence of a type IIb Na-Pi cotransporter in the RNA samples collected, reverse transcription PCR (**RT-PCR**) was performed. First, strand cDNA was synthesized by Moloney murine leukemia virus reverse transcription (Promega Corp., Madison, WI) using random primers (Promega Corp.) in the presence of Recombinant RNasin Ribonuclease Inhibitor (Promega Corp.). The PCR was completed using a pair of primers, which were designed based on a partial sequence of a chicken intestinal Na-Pi IIb cotransporter described by Werner and Kinne (2001; GenBank, AF297187), with the sense primer corresponding to bp 60 to 79 and antisense primer from bp 84 to 203. The sequence of the sense primer was 5′-GGGAACAATCCATGATTTC, and the antisense primer was 5′-GTAGCTCAGGGGCATCTTCA-3′ producing a 144-bp product. This product was cloned and sequenced as described below.

Rapid Amplification of cDNA Ends

To obtain the full-length cDNA, RNase-ligase-mediated rapid amplification of cDNA ends PCR was performed using the GeneRacer Kit (Invitrogen Life Technologies, Carlsbad, CA). This procedure allows for the cloning of gene segments in either the 5′ or 3′ direction from a region of known sequence. Briefly, mRNA was decapped, ligated to a GeneRacer RNA Oligo at the 5′ end, and then reverse-transcribed using SuperScript II RT and a GeneRacer Oligo dT primer to obtain a rapid amplification of cDNA ends-(**RACE**) ready first-strand cDNA. The 5′-RACE PCR product was amplified by using a reverse gene-specific primer and the GeneRacer 5′ primer (homologous to the GeneRacer RNA Oligo), and the 3′-RACE PCR product was obtained by amplifying the first-strand cDNA using a forward gene-specific primer and the GeneRace 3′ primer (homologous to the GeneRacer Oligo dT primer). The gene-specific primers were designed according to guidelines provided in the kit instruction manual (L1502-2, Invitrogen Life Technologies), again based on the partial

sequence described by Werner and Kinne (2001). The reverse gene-specific primer was positioned at 175 to 200 bp, and the sequence was 5′-GCTCAGGGGCATCTTCACCACTTTCA-3′; the forward gene-specific primer was at 499 to 526 bp, and the sequence was 5′-TGCCATCGGTCTCATACTTCTGGCTTTG-3′.

Cloning

The initial PCR, 5′-, and 3′-RACE products were visualized by electrophoresis, gel-purified using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA), and then subcloned into pCR4Blunt-TOPO plasmid vector using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen Life Technologies). The cloned PCR products were sequenced on both strands using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM, Applied Biosystems, Foster City, CA) with an ABI PRISM 310 genetic analyzer.

Northern Blot

Total RNA was extracted from the broiler heart, breast muscle, liver, brain, lung, kidney, pancreas, duodenum, jejunum, and ileum using Trizol reagent. Ten micrograms of total RNA from each tissue was fractionated by 1% agarose gel electrophoresis and transferred to a Zeta-Probe GT blotting membrane (Bio-Rad Laboratories, Hercules, CA) and ultravioletly crosslinked (UV Stratalinker 2400, Stratagene Cloning Systems, La Jolla, CA). Each loading and transfer of total RNA was confirmed by detection of 18S and 28S rRNA on the blot after transfer under ultraviolet light. The membrane was prehybridized in PerfectHyb Plus hybridization buffer (Sigma-Aldrich Corp., St. Louis, MO) at 55°C for 10 min. The cDNA probe was labeled with [^{32}P] dCTP using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membrane was hybridized overnight at 55°C and washed once with 2XSSC, 0.1% SDS for 10 min at room temperature, and then twice with 0.5XSSC, 0.1% SDS for 10 min each at 55°C. The membrane was then wrapped in plastic wrap and exposed to a phosphor storage screen overnight and scanned using a Typhoon phosphorimager (Amersham Biosciences, Buckinghamshire, UK). To determine the relative expression level of the gene in different tissues, the Northern blot signals were quantified using ImageQuant software (Amersham Biosciences) and adjusted by the actual amount of total RNA transferred onto the blotting membrane, which was estimated by quantifying the ethidium bromide staining intensities of the 28S and 18S rRNA on the membrane after ultraviolet crosslinking and before hybridization as determined by scanning with the Typhoon and quantification by ImageQuant.

In Situ Hybridization

Complementary RNA probes were synthesized from the linearized pCR4Blunt-TOPO plasma containing the cDNA fragment (about 850 bp) of Na-Pi IIb by in vitro transcription in the presence of digoxigen-labeled UTP (Boehringer Mannheim GmbH, Mannheim, Germany), using the MAXIscript T_7/T_3 Kit (Ambion, Austin, TX). Depending on the orientation of the insert, either T_3 or T_7 RNA polymerase was used to generate an antisense or sense probe (used as negative control). A piece of jejunum was fixed, embedded in paraffin, sectioned, and placed on microscope slides under RNase-free conditions. Slides were rehydrated by immersing in xylene (twice, 10 min each) and ethanol (100% ethanol for 10 min, 70% ethanol for 10 min), washed by deionized water (3 min) and PBS (15 min), digested by proteinase K (2 μg/mL) for 15 min at 37°C, and acetylated for 10 min with 0.25% acetic anhydride in 0.1 *M* triethanolamine. The cRNA probes were added and hybridized to the tissue sections at 50°C for 16 h in a humid CMT hybridization chamber (Corning, Inc., Corning, NY). Before hybridization, the chamber was subjected to 65°C for 15 min to block endogenous alkaline phosphatase activity. After hybridization, sections were washed with 2XSSC, 0.1% SDS for 4 times for 5 min each at room temperature; 0.1XSSC, 0.1% SDS for 2 times for 10 min each at 42°C; rinsed with 2XSSC and then treated with RNase I (100 μg/μL) at 37°C for 15 min; and rinsed with 2XSSC again. The hybridization signal was detected with a DIG Nucleic Acid Detection Kit (Boehringer Mannheim GmbH). Levamisole was added to the color reaction at 0.1 μM to block the endogenous alkaline phosphatase activity. The color reactions were left in the dark at room temperature for 15 min and then checked under microscope periodically. When desired darkness of the signals was achieved, slides were placed in a stop buffer (5 m*M* EDTA, 20 m*M* Tris, pH = 7.5) for at least 5 min to stop the reactions. To mount the slides, 100 μL of PBS:glycerol (1:1) was added to each slide when the slides were still wet. Slides were then covered with glass cover slips and dried at room temperature. Signals were observed, and microscopic images were collected.

Dietary Manipulation

All animal work was approved by the University of Maryland Animal Care Committee. To determine the effects of dietary P on the expression of the chicken intestinal Na-Pi cotransporter, experimental diets were formulated to be deficient in total P. Ross 308 chicks (n = 160) were fed either a control diet consisting of 1.2% Ca and 0.55% nonphytate P (**nPP**) or a restricted diet containing 0.6% Ca and 0.25% nPP from hatch to 4 d of age. These diets were formulated based on a corn and soybean meal mash basal diet formulated to meet NRC (1994) broiler requirements for all nutrients except Ca and P (Table 1). The basal diet was analyzed for P colorimetrically (Heinonen and Lahti, 1981) and Ca by atomic absorption (Perkin-Elmer, 1982). Based on analyzed values, appropriate amounts of calcium carbonate, monocalcium phosphate, and Celite (Seegott Inc., Streetsboro, OH, used as an indigestible marker and filler at a minimum inclusion of 1%) were added to the basal diet to provide the test diets with the desired concentrations of P and Ca. The ratio of Ca to P was maintained across both diets due to prior observations

Table 1. Composition of the basal diet

Ingredients	Starter basal, g/kg
Yellow corn	585.89
Soybean meal	342.61
Soy oil	30.00
Corn gluten meal	22.22
Salt	5.92
Calcium carbonate	4.33
Monocalcium phosphate	4.04
DL-Met	2.14
Lys HCl	0.75
Southern States mineral mix[1]	0.70
Vitamin premix[2]	0.70
Choline chloride, 60%	0.70
Calculate (analyzed) nutrients	
DM, %	89.7 (89.6 ± 0.001)
ME, kcal/kg	3,144
CP, %	22.7
Ca, %	0.41 (0.39 ± 0.004)
P, %	0.47 (0.49 ± 0.004)
nPP,[3] %	0.21 (0.21 ± 0.004)

[1]Provides the following per kilogram of diet: Zn (from ZnO), 147 ppm; Mn (from $MnSO_4$), 84 ppm; Fe (from $FeSO_4$), 28 mg; Cu (from CuO), 14 mg; I (from $CaIO_3$), 2.1 ppm; Co (from $CoCO_3$), 0.035 mg; Southern States Coop., Salisbury, MD.

[2]Provides the following per kilogram of diet: vitamin A (from retinyl acetate), 13,135 IU; cholecalciferol, 4,636 ICU; vitamin E (from DL-α-tocopheryl acetate), 46.4 IU; vitamin B_{12} (from cyanocobalamin), 0.023 mg; riboflavin (from riboflavin), 15.5 mg; niacin (from nicotinic acid), 61.8 mg; pantothenic acid (from calcium D-pantothenate), 21.6 mg; vitamin K_3 (from menadion sodium bisulfite complex), 2.78 mg; folic acid (from folic acid), 1.85 mg; pyridoxine (from pyridoxine hydrochloride), 5.41 mg, thiamin (thiamine mononitrate) 3.86 mg; biotin (from D-biotin), 0.124 mg.

[3]nPP = nonphytate phosphorus; determined by subtracting analyzed phytate P from analyzed P.

noting the relationship between Ca and P absorption (al-Masri, 1995). As Ca:P increases, there is a significant decrease in P absorption and retention; therefore, to minimize this effect, Ca:P was kept constant. The birds were housed at 10 birds per pen and 8 pens per treatment. Birds were euthanized by cervical dislocation and ileal contents collected for nutrient retention. One bird per pen was selected at random, and the duodenum, jejunum, and ileum were collected and RNA extracted as described previously. For consistency of comparison across individuals, the anatomical location of samples collected was maintained across birds by taking tissue for analysis from a specific site in the intestine that was divided into the 3 regions using the following landmarks: duodenum (from distal of the gizzard to 1 cm distal of the bile duct), jejunum (1 cm distal from the bile duct to the Meckel's diverticulum), and ileum (Meckel's diverticulum to 5 cm proximal to the ileocecal junction). The duodenal section was collected as a 1-cm segment in the duodenal loop. The jejunal sample was collected as a 1-cm section at the center of the jejunal segment. The ileal sample was collected as a 1-cm section at 5 cm distal to the Meckel's diverticulum.

Real-Time PCR Detection of Chicken Na-Pi IIb Cotransporter Gene Expression

Gene expression measurements for the chicken Na-Pi IIb cotransporter were determined using real-time quantitative PCR. Primers for the Na-Pi cotransporter mRNA were designed using Beacon Designer software for SYBR Green detection (Premier Biosoft, Palo Alto, CA). Real-time PCR was carried out using the Bio-Rad iQ instrument and with the iScript and iQ-SYBR Green Supermix kits, using the manufacturers protocols (Bio-Rad Laboratories). Individual cDNA was diluted 1:20 before amplification. Thermocycling parameters were as follows: 94°C for 5 min; 50 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 8 min. Fluorescence measurements were collected at every cycle during the extension step (72°C). The primers used included Na-Pi IIb FOR 5′-CTGGATGCACTCCCT AGAGC-3′, Na-PiIIb-REV5′-TTATCTTTGGCACCCTCC TG-3′, ch18s-FOR 5′-CCGAGAGGGAGCCTGAGAA-3′, and ch18s-REV 5′-CGCCAGCTCGATCCCAAGA-3′. Each gene was amplified independently in triplicate within a single instrument run. Standard curves were also run to determine the efficiency of amplification by pooling undiluted cDNA from the duodenum samples across both treatments and diluting the pooled cDNA to dilutions of 1:5, 1:20, 1:100, and 1:500. Gene expression was normalized for RNA loading using 18S rRNA as an internal control, and differences in gene expression were determined using the ΔCt method of Pfaffl (2001) including a correction for amplification efficiencies.

RESULTS AND DISCUSSION

Molecular Properties of Na-Pi IIb Cotransporter

Reverse transcription PCR was performed with the pair of primers designed, based on a partial sequence of chicken intestinal Na-Pi IIb cotransporter cDNA (GenBank, AF297187). A PCR product of predicted size (144 bp) was obtained, indicating that this Na-Pi cotransporter was present in the broiler intestine. Preliminary analysis with RT-PCR also suggested that the cotransporter was expressed at a higher level in the duodenum than in the ileum (data not shown). To obtain the full length of this cDNA, we performed RNase-ligase-mediated rapid amplification of cDNA ends. Both 5′ and 3′ PCR products of RACE were subcloned and sequenced on both strands. The nucleotide and predicted amino acid sequence of the type IIb Na-Pi cotransporter is shown in Figure 1. The cDNA contains an open reading frame of 2,022 nucleotides, and it encodes a protein of 674 amino acids with the calculated molecular mass of 73,719 Da. The amino acid sequence of the Na-Pi IIb cotransporter has relatively high homology with other type II Na-Pi cotransporters, including the human IIb (66.1%), mouse IIb (65.3%), zebrafish IIb (67.6%), flounder IIb (68.8%), human IIa (65.2%), mouse IIa (64.9%), and bovine IIa (62.3%). Alignment of the broiler Na-Pi IIb sequence with the human IIb (GenBank, AF146976), mouse IIb (GenBank, AF081499), flounder IIb (GenBank, PAU13963), and zebrafish IIb (GenBank, AF297180), using the T-Coffee software (http://www.ch.Embnet.org/software/TCoffee.html), is shown in Figure 2. The homology of this Na-Pi IIb cotransporter with the type I or type III

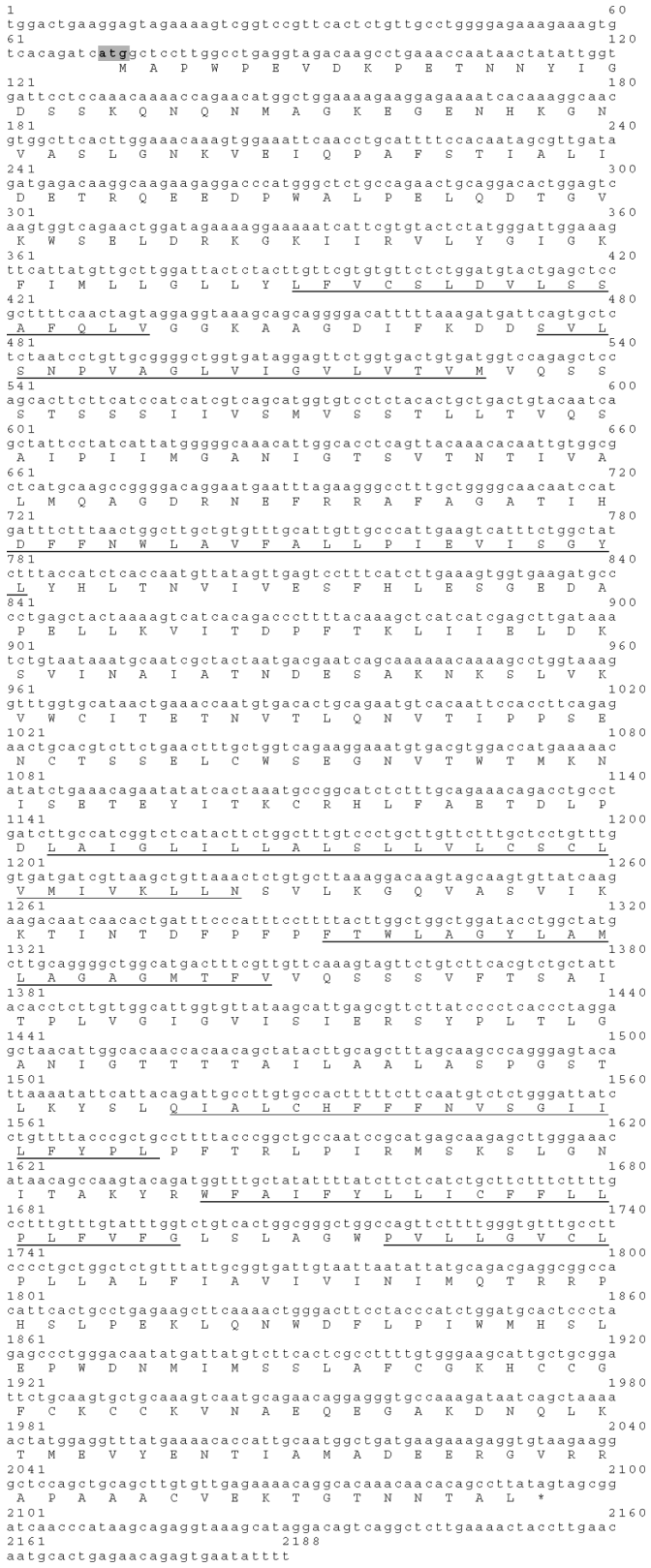

Figure 1. Nucleotide (top) and predicted amino acid (bottom) sequence of the broiler intestinal Na-organic phosphate (Pi) IIb cotransporter. The stop codon at nucleotide 2094 (tag) is indicated by an asterisk. Predicted 8 transmembrane domains are underlined [fitted by comparing with the topological data of other Na-Pi cotransporters (Lambert et al., 1999; Paquin et al., 1999; Graham et al., 2003)] and analyzed with the TMHMM software (http://www.cbs.dtu.dk/services/TMHMM-2.0).

Na-Pi cotransporters is relatively low (around 20%). The fact that the broiler Na-Pi IIb has high homology with the type II Na-Pi cotransporters and low homology with the type I or type III cotransporters indicates that it belongs to the type II family. As a characteristic of the type II Na-Pi cotransporter, the COOH terminus contains a conserved PSD95-Dlg-zona occludens-1 (**PDZ**) binding motif, N-T-A-L (A/X-T-X-L/F). The PDZ binding motif of the type IIa Na-Pi cotransporter has been found to affect the apical expression of the transporter by interacting with several PDZ proteins (Karim-Jimenez et al., 2001), which are known to interact with the actin cytoskeleton and therefore might contribute to stabilizing proteins on the plasma membrane (Fanning and Anderson, 1996). A stretch of cysteines is also present near the COOH terminus, a characteristic of Na-Pi cotransporters of type IIb and absent in type IIa, further indicating that this broiler Na-Pi contransporter belongs to the type IIb subfamily. Eight transmembrane domains were predicted by comparison with topological data from other Na-Pi cotransporter sequences (Lambert et al., 1999; Paquin et al., 1999; Graham et al., 2003) and analysis with TMHMM prediction software (http://www.cbs.dtu.dk/services/TMHMM-2.0). Both NH_2 and COOH termini are predicted to be intracellular.

Analysis of the broiler type IIb Na-Pi cotransporter identified 6 potential consensus sequences for N-linked glycosylation (N-X-S/T located at Asn-292,305,310,318,330, and 337) within a large putative extracelluar loop. The amino acid sequence alignment of broiler, human, mouse, flounder, and zebrafish Na-Pi IIb cotransporters (Figure 2) indicated that 4 N-linked glycosylation sites at 292, 310, 318, and 337 are conservative among the 5 species. The Asn-305 site is present in all species except zebrafish, and site Asn-330 is present only in flounder other than in the broiler. Two N-linked glycosylation sites of the rat renal type IIa Na-Pi cotransporter, located at Asn-298 and Asn-328, have been confirmed to be glycosylated, and the glycosylation is likely important for apical membrane expression (Hayes et al., 1994). In mouse, it has also been shown that the intestinal type IIb Na-Pi cotransporter is a glycoprotein and that the glycosylation is age-dependent (Arima et al., 2002).

This broiler Na-Pi IIb cotransporter has slightly higher homology with flounder (68.8%) and zebrafish (67.6%) than with human (66.1%) and mice (65.3%) Na-Pi IIb cotransporters. Although the type IIa cotransporters were identified earlier and studied more extensively than the type IIb, the type IIb Na-Pi are actually present in more species than is the type IIa. The type IIb Na-Pi cotransporters are expressed in almost all vertebrates, whereas expression of the type IIa Na-Pi cotransporters are restricted, at least based on current knowledge, to mammals and chickens. It is suggested that the type IIb is a more ancient isoform, and the type IIa evolved with the development of the mammalian-type nephron (Werner and Kinne, 2001), likely for more specialization for renal Pi reabsorption.

Tissue-Specific Expression

Northern blot analysis was performed to characterize the Na-Pi IIb cotransporter expression (Figure 3). A single

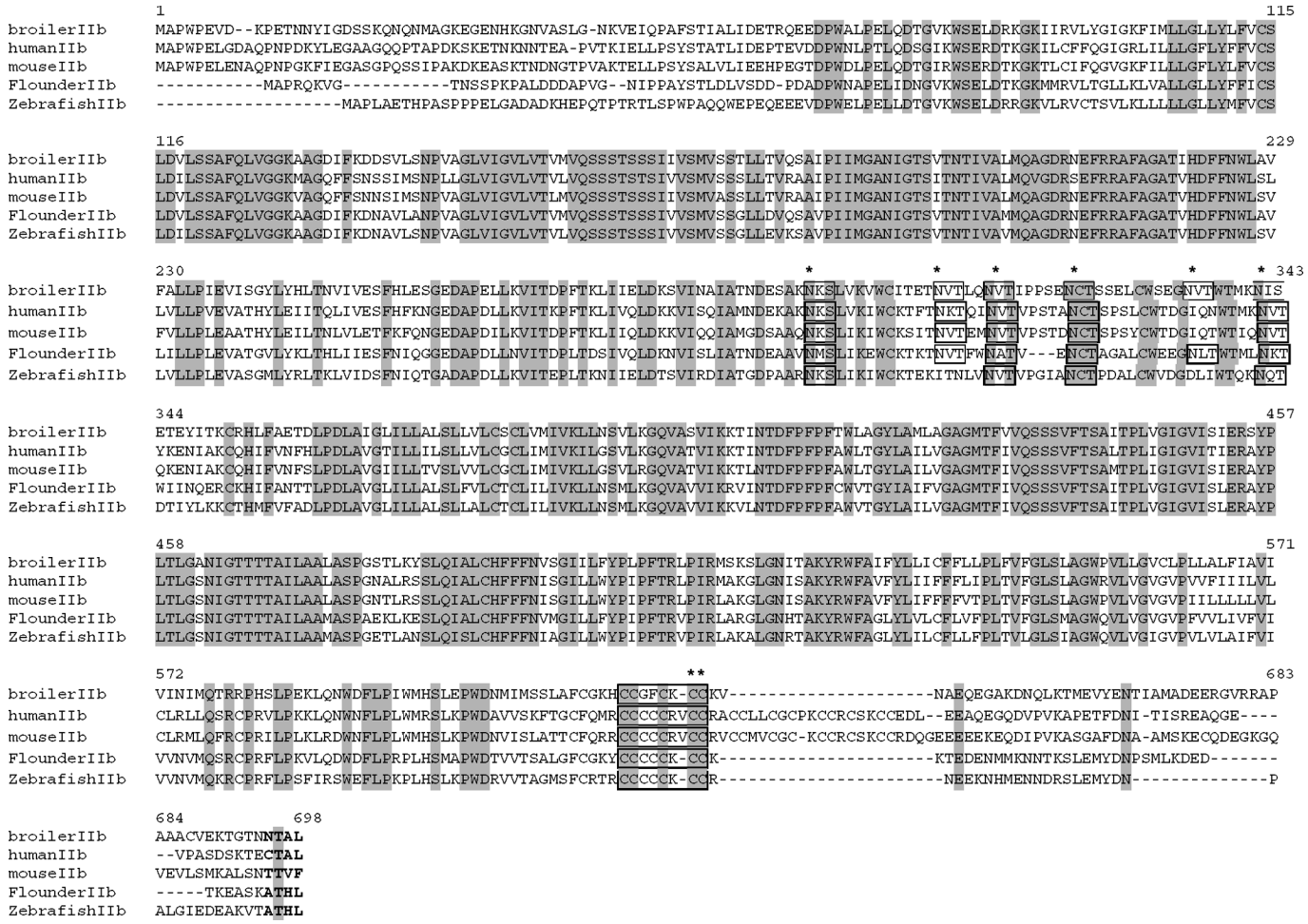

Figure 2. Amino acid sequence alignment of the broiler Na-inorganic phosphate (Pi) IIb sequence with that of the human IIb (GenBank, AF146976), mouse IIb (GenBank, AF081499), flounder IIb (GenBank, PAU13963), and zebrafish IIb (GenBank, AF297180). The multiple sequence alignment was made using T-Coffee (http://www.ch.Embnet.org/software/TCoffee.html). Amino acids that are fully conserved are highlighted. The COOH terminal conserved PSD95-Dlg-zona occludens-1 (PDZ) binding motif is bolded. *Amino acids with putative N-linked glycosylation are boxed. **The cysteine stretch (characteristic of the type IIb Na-Pi cotransporter family) is boxed. Numbering of amino acids is based on the mouse sequence.

transcript was observed, and among the ten tissues tested (heart, muscle, liver, brain, lung, kidney, pancreas, duodenum, jejunum, and ileum), the transporter is predominantly expressed in the small intestine tissue (the duodenum, jejunum, and ileum). Signals were also detected in the lung and very weakly in the brain. No hybridization signal was detected in the heart, muscle, liver, kidney, and pancreas. Relative quantification of the northern blot signals revealed that, among the 3 sections of the small intestine, the gene is expressed the highest in the duodenum, followed by the jejunum, and is lowest in the ileum, with the signal in the duodenum being about 2.5 times that in the jejunum, and the signal in the jejunum being about 2.5 times that in the ileum (Figure 4). This agrees with our preliminary results obtained with RT-PCR, which showed that the gene is expressed at a higher level in the duodenum than in the ileum. The jejunum has been considered to be the major site for Pi absorption in chicks (Hurwitz and Bar, 1970; Blahos and Care, 1981). The duodenum, however, may have a greater ability to absorb Pi than the jejunum, but its short length and short transit time of digesta through the duodenum possibly limits its role in overall intestinal Pi absorption. Among the other tissues (heart, muscle, liver, brain, lung, kidney, and pancreas) tested, this Na-Pi cotransporter can be detected only in the lung and possibly the brain. However, in humans it was shown that the type IIb Na-Pi cotransporter is expressed in many tissues, including the lung, small intestine, kidney, prostate, pancreas, and mammary gland, among others, with the highest expression found in the lung (Xu et al., 1999). In mice, the type IIb Na-Pi cotransporter was found to be expressed in the mucosa of the upper small intestine, colon, liver, lung, kidney, and testis (Hilfiker et al., 1998). It is unclear why the broiler type IIb Na-Pi cotransporter was found not to be expressed in as many tissues as it is in humans and mice. It is possible that other isoform(s) of type IIb Na-Pi might be present in other tissues and that the isoform identified here is more specific to the small intestine. Most fish species (shark, skate, trout, and zebrafish) have been shown to have 2

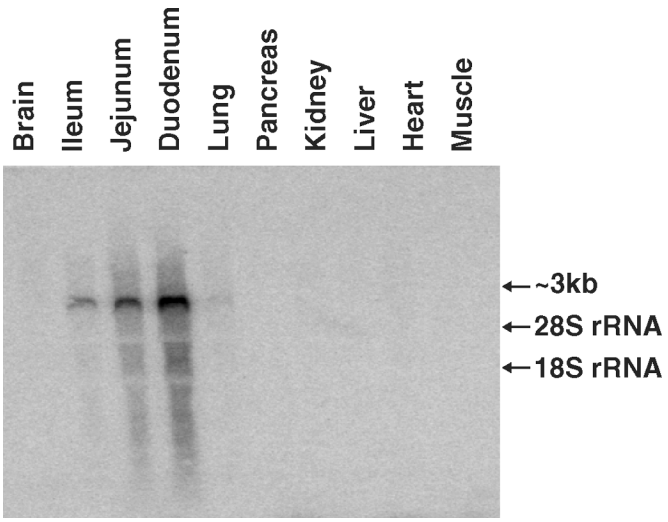

Figure 3. Northern blot analysis of broiler Na-inorganic phosphate (Pi) IIb in broiler brain, ileum, jejunum, duodenum, lung, pancreas, kidney, liver, heart, and muscle. Ten micrograms of total RNA was loaded for each lane from an individual bird. Hybridization was carried out at 55°C for 16 h. The membrane was washed once with 2XSSC, 0.1% SDS for 10 min at room temperature and then twice with 0.5XSSC, 0.1% SDS for 10 min each at 55°C. The hybridization pattern is indicative of that observed in replicate blots of RNA extracted from multiple birds (n = 3). rRNA = ribosomal RNA.

isoforms of the type IIb Na-Pi cotransporters (Werner and Kinne, 2001; Graham et al., 2003).

Cell-Specific Expression

In situ hybridization done using a section of jejunum showed that the type IIb Na-Pi cotransporter mRNA seemed to be homogeneously expressed in the epithelial cells from within the crypt area to the villus tips (Figure 5). Immunohistochemistry, using a monoclonal antibody, indicates that the type IIb Na-Pi cotransporter protein is located at the brush-border membrane of enterocytes in mice (Hilfiker et al., 1998; Hattenhauer et al., 1999). More specifically, the mouse type IIb Na-Pi cotransporter is only present along the villi and absent in the enterocytes of the crypt area (Hattenhauer et al., 1999). In our study, in situ hybridization could only detect the mRNA of the type IIb Na-Pi cotransporter and did not reveal where the cotransporter protein is located. Development of a monoclonal or polyclonal antibody specific to the broiler type IIb Na-Pi cotransporter protein is needed to locate the transporter protein as well as to characterize its function in intestinal Pi absorption and to study its role in the intestinal adaptation to altered P conditions in the body.

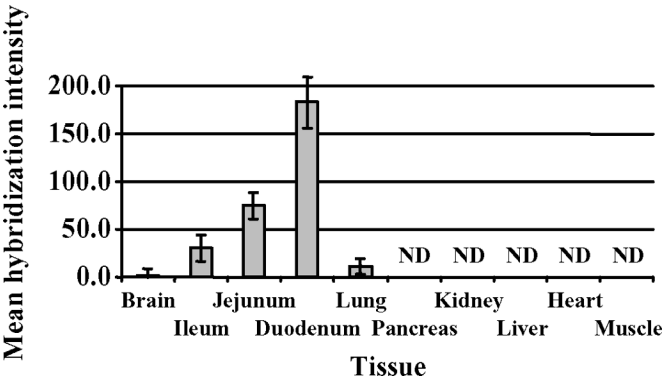

Figure 4. Relative quantification of Na-inorganic phosphate (Pi) IIb gene expression (Na-Pi IIb/RNA × 100) in different tissues tested with Northern blot analysis. Mean hybridization signal intensity (±SD) was quantified using the Typhoon phosphoimager (Amersham Biosciences, Buckinghamshire, UK) and ImageQuant software (Amersham Biosciences). ND = none detected > background.

Genomic Localization

Once the chicken whole genome draft sequence produced by the Washington University Genome Sequencing Center (St. Louis, MO) was released, several genome analysis centers began to annotate the data. Using the Ensembl genome browser (http://www.ensembl.org), we were able to query the genome sequence using the cloned cDNA sequence for the IIb Na-Pi cotransporter. This gene is located on chicken chromosome 4 (GGA4) at 76.3 Mb, and its highly conserved genomic structure consists of 12 exons. Putative orthologs are found in many other genomes including human (chromosome 4 at 25.35 Mb) and mouse (chromosome 5 at 51.7 Mb).

Quantitative trait loci have been previously mapped in chickens to the region of GGA4 where the cotransporter gene resides. The most prominent of these studies was the mapping of QTL for production traits in egg layers, which described QTL for egg weight, egg number, and BW near the cotransporter gene (Tuiskula-Haavisto et al., 2002). A similar finding was reported for BW in a broiler Leghorn cross by Sewalem et al. (2002). The orthologous gene locus in human has been associated with bone mineral density, based on results from a large clinical study, but only in males (Karasik et al., 2003). In mice, a QTL for bone mineral density was also detected in the IIb Na-Pi cotransporter orthologous genome region (Beamer et al., 1999). These data support the role of the IIb Na-Pi cotransporter as a positional candidate gene for P absorption in the intestine and traits limited by P uptake, including bone mineralization, egg production, and BW.

Response to Dietary P

The response of the chicken intestinal Na-Pi IIb cotransporter to variation in substrate levels (dietary P) was determined by feeding newly hatched chicks a diet deficient in nPP, followed by the measurement of the gene expression level of the cotransporter mRNA using real-time PCR. The effect of dietary treatment was determined by analyzing the resulting Ct values for each amplification and determining the ΔCt using the level of 18S rRNA as an internal standard for normalizing the amount of RNA in each reaction. The average Ct values for each of the tissues and treatments are shown in Table 2. The differences in Ct values for gene expression were determined to be significantly different using a Student's *t*-test, and the relative

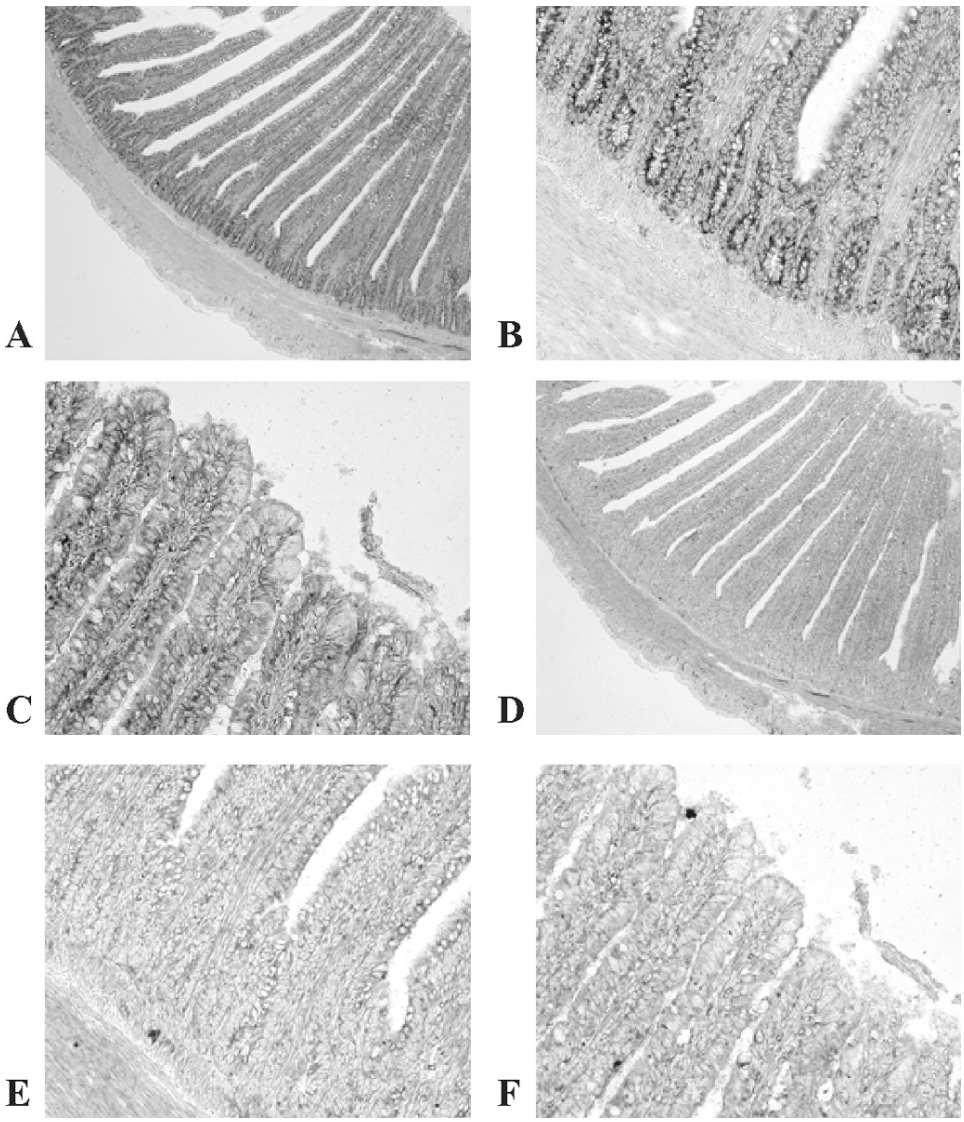

Figure 5. In situ hybridization of type IIb Na-inorganic phosphate (Pi) cotransporter expression in 28-d-old broiler jejunum. Sections of jejunum were hybridized with antisense (A, B, C) and sense (D, E, F) digoxigen-labeled cRNA probes for the Na-Pi IIb cotransporter. The black color indicates specific hybridization. The signal was detected throughout the crypt-villi axis with antisense probes, with no signal observed with sense probes. A and D = section, 80× magnification; B and E = crypts, 400× magnification; C and F = villi, 400× magnification.

difference in expression caused by the dietary treatment is also reported in Table 2.

The reduction of P in the diet had a significant effect on the expression of the Na-Pi cotransporter by stimulating an average 2.8-fold increase in the mRNA levels in the small intestine. Nearly identical stimulatory effects were seen across all segments of the intestine, with relative expression decreasing from duodenum to ileum. This pattern is similar to that reported in trout, in which a 2-fold induction of the Na-Pi IIb transporter was observed as a result of a 40% reduction of dietary P (Sugiura et al., 2003) and is in the range of expression induction reported in mice for the Na-Pi IIb cotransporter as a result of feeding a low-P diet (Segawa et al., 2004). The effect of reducing dietary P from 0.50% in the control diet to 0.25 in the low-P diet induced Na-Pi IIb expression an average of 2.3-fold across all small intestinal segments. This change in expression was shown not to be involved in the vitamin D signaling pathway, because knockout mice showed a similar response to that of wild-type mice (Segawa et al., 2004). The influence of vitamin D on the absorption of Pi has long been known (Favus, 1985). Recent work with knockout mice has shown that 25-hydroxyvitamin-D_3-1α-hydroxylase, which leads to an increase in the level of 1,25-dihydroxyvitamin D_3, does not influence the expression of the Na-Pi IIb cotransporter in the intestine (Capuano et al., 2005). Therefore, the regulation of expression of the Na-Pi IIb cotransporter by dietary P must function through a novel pathway and not one directly involving vitamin D.

In conclusion, we have cloned and sequenced a Na-Pi IIb cotransporter from the broiler small intestine. It has an open reading frame of 2,022 bp and encodes a protein of 674 amino acids with a calculated molecular weight of 74 kDa. This type IIb Na-Pi cotransporter is almost exclusively expressed in the small intestine, with the highest expression in the duodenum, followed by the jejunum,

Table 2. Effect of the low-P diet on gene expression

Dietary treatment	Tissue	Gene expression[1] 18S rRNA	Gene expression[1] Na-Pi IIb mRNA	n-Fold[2]
Control[3]	Duodenum	15.8 ± 0.2[a]	23.2 ± 0.2[a]	1.0
	Jejunum	16.1 ± 0.3[a]	24.4 ± 0.3[a]	1.0
	Ileum	16.2 ± 0.3[a]	25.3 ± 0.4[a]	1.0
Low P[3]	Duodenum	16.0 ± 0.3[a]	24.5 ± 0.3[b]	3.1
	Jejunum	16.2 ± 0.3[a]	26.1 ± 0.4[b]	2.9
	Ileum	16.2 ± 0.4[a]	26.9 ± 0.2[b]	2.5

[a,b]Means with common superscripts do not differ significantly between the treatments ($P < 0.05$).

[1]Ct values are the average ± SD (n = 8 birds per treatment); rRNA = ribosomal RNA; Pi = inorganic phosphate.

[2]n-Fold change in gene expression was calculated by the ΔCt method of Pfaffl (2001) including the amplification efficiency for both genes; 18S amplified with 94% and Na-Pi IIb amplified with 91% efficiencies, respectively, as determined by the standard curves of diluted cDNA. Expression levels for the control diet were set as 1.0 for fold change effect determination within a tissue.

[3]Diets were formulated to contain 1.2% Ca and 0.55% nonphytate P (nPP) or a restricted diet (Kow P) containing 0.6% Ca and 0.25% nPP from hatch to 4 d of age. The Ca:P was maintained to eliminate the negative effects of high ratios on P absorption and retention (al-Masri, 1995).

and then the ileum. The mRNA of the type IIb Na-Pi cotransporter is detected in both villi and crypts of the jejunum. The expression of the gene responds to dietary P concentration changes by increasing expression when P is reduced. Its role in the absorption of Pi and adaptation to altered P conditions in the body needs to be further characterized. The genomic localization of the gene provides support for the type IIb Na-Pi cotransporter to be a positional candidate gene underlying production trait QTL.

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